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MINERVA BIOTEC 2014;26:149-57

Using CM11 peptide as a cell permeable agent for the improvement of conventional plasmid transformation methods in *Escherichia coli* and *Bacillus subtilis*

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Aim. **Cold calcium chloride is the most practical and cheapest method for transformation of DNA into bacteria such as *Escherichia coli*, while some bacteria such as *Bacillus subtilis* uptake DNA in natural way. This study reports the increase of transformation efficiency using cationic peptide as a novel and efficient agent to transport DNA plasmids into Gram-negative and positive bacteria.**

Methods. **pET-28a(+), pGEX4T-1 and pUC19 plasmids were separately transferred into *E.coli*, also *B.subtilis* was transformed by pWB980 plasmid. $CaCl_2$ treated *E.coli* competent cells were prepared in the presence of different concentration of CM11 peptide in different time and mix with adequate amount of plasmids. In the case of *B. subtilis,* natural spontaneous DNA transformation accompanied with presence of different concentration of peptide.**

Results. **In the presence of peptide, the transformation yield of pET-28a(+), pGEX4T-1 and pUC19 plasmids was 4.7, 4.4 and 4 fold higher than control, respectively. Results were also statistically significant p-value was less than 0.001 for all plasmids. Also the entry of pWB980 plasmid into *B. subtilis* with $P<0.001$ improved efficiently which was 6.4 fold higher than basal conditions.**

Conclusion. **These studies showed that CM11 peptide as a cell permeable peptide can increase the plasmid transformation efficiency in bacterial cells.**

Key words: Peptide - Transformation, calcium chloride - Spizizen - Transformation.

DNA Transformation is the main step of gene cloning in the bacterial hosts. The most bacterial strains are able to uptake DNA molecule from the surroundings but the uptake ability is not the same for all strains. *Escherichia coli*, which is the most applicable host in comparison with other bacteria, in normal conditions has a little ability to uptake DNA and the possibility of being transformed is not desirable for gene cloning.[1, 2] Hence, to optimize the amount of the transformation event, bacteria should be under certain treatments in order to increase the uptake ability. Before 1970 the efforts for DNA transformation into bacterial cells were unsuccessful until Mendel and Hyuga found that cold calcium chloride causes the bacteria to take up the DNA phage.[3] Followed by 1972, Cohen showed that calcium chloride is also effective for DNA plasmid transformation.[4] Cold calcium chloride method is one of the most practical and cheapest methods to transform combinant DNA into the Gram-negative bacteria cells but due to cell structure of Gram-positive bacteria this method is not applicable for these bacteria. Calcium chloride causes that DNA connect to the external surface of the bacterial cell membrane. In this state, due to presence of DNA on the surface of target cell, capability for DNA uptake will be increased significantly. Subsequently heat shock will be applied to transform the accumulated DNA into the cytoplasm.[5] In this procedure two points

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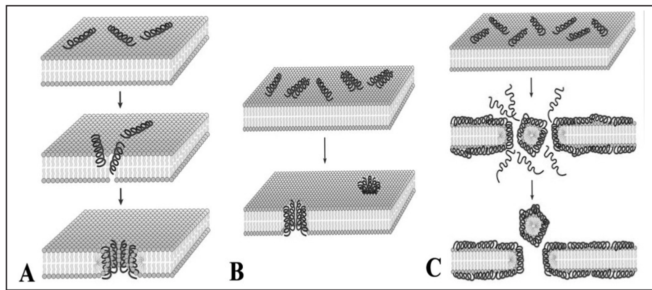

Figure 1.—Three models according to which the cationic peptides influence and change the structure of bacterial cell membranes: Barrel-stave model (A), Toroidal model (B) and Carpet model (C).

are notable: first, accumulation of foreign DNA on bacterial cells increases by calcium chloride, second, permeability of cell membrane increases by heat shock. Therefore, it seems that achieving to a compound being able to increase efficiency of each of these two steps could be effective in optimization of this method. In Gram-negative bacteria such as *E.coli,* lipopolysaccharide (LPS) structure in outer membrane acts as one of the major entry barriers of free compounds such as DNA.[6] Lipopolysaccharide has polyanionic structure which is stable on cell surface in cause of dual bridges created elements such as Mg^{2+}cation. Studies have shown that polycationic compounds with positive charge are able to compete with these elements and eliminate them. It causes to form an imitative cationic bridge in the LPS surface that leads instability and permeability in the bacterial cell membrane.[7, 8] During these years, considerable researches have been done on cell permeable peptides (CPPs) as a tool to deliver and release drugs and other biologic compounds that are not able to transfer into target cell. These peptides are composed of 20 to 50 amino acids and their sequences are positively charged due to the large number of arginine and lysine amino acids. They can pass through cell membrane by their amphipathic and hydrophobic structures without need to membrane receptor. Therefore, these peptides can be used for transferring a wide range of biologically active molecules such as proteins and oligonucleotides.[9, 10] In Gram-negative bacteria, CPPs with positive charge by binding to anionic groups in outer membrane structure such as phospholipids phosphate groups and in Gram-positive bacteria by binding to surface teichoic acids in cell membrane, could penetrate into cell membrane and create the pores. The net results of this phenomenon are instability and permeability in bacterial cells.[11, 12] Studies have shown that changes in the structure of cell membrane and pore formation are based on three models: Barrel-stave, Torodial, Carpet (Figure 1). For each model, changes depend on the amount of positive charge, size, and distribution of hydrophilic and hydrophobic regions, combinations of amino acids constituting the peptide. however, in all models, CPPs first interact with the negatively charged lipid-head groups of the cytoplasmic membrane, they accumulate facing parallel to the lipid bilayer until a critical threshold concentration is reached, after which they self-organize to form a permeation pathway.[13, 14] Cecropin and Melittin are some peptides with considerable ability to influence on the structure of bacterial membranes. Cecropin peptides are composed of 37-39 amino acids, which have an extremely amphipathic N-terminal with α-Helix structure attached to the hydrophobic C-terminal through an elastic part.[15] Melittin peptide is also composed of 26 amino acids that unlike cecropin have an extremely amphipathic C-terminal with α-Helix structure and hydrophobic N-terminal.[16] In this study, the effect of CM11 peptide (WKLFKKILKVL-NH_2) composed of Cecropin (1-7 residues) - Mellitin (5-8 residues), [17, 18], peptides was analyzed as a membrane permeable peptide for improvement of chemical and natural DNA transformation in *E. coli* and *B. subtilis*, respectively. For this purpose, improvement of transformation efficiency mediated by CM11 was analyzed via transformation of pET-28a (+), pGEX4T-1 and pUC19 plasmids into *E. coli* and pWB980 plasmid into *B. subtilis*.

Materials and methods

Peptide synthesis

The CM11 hybrid peptide was synthesized as a C-terminal carboxamide on a Rink p-methylbenzhydrylamine resin by the solid-phase using standard method.[19] The peptide was purified by reverse-phase semi preparative HPLC on C18 Tracer column using a linear gradient from 10 to 60% acetonitrile in water with 0.1% trifluoroacetic acid over 50 min. The peptide was obtained with >95% HPLC purity. Electrospray ionization mass spectrometry was used to confirm peptide identity.[19]

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Bacteria strains and plasmids

Escherichia coli BL21 (DE3) pLysS (Invitrogen, USA) and *Bacillus subtilis* WB600 were used as host for plasmids transformation. The pET28a (+) (5.4 kb), pGX4T-1 (4.9 kb) and pUC19 (2.7 kb) plasmids were used to transfer into *Escherichia coli* BL21 and pWB980 (3.8 kb) plasmid used for *Bacillus subtilis* (Amersham, England).

Peptide soluble preparation

The peptide was dissolved in phosphate–buffered saline (pH 7.2), yielding 1mg/mL stock solution.

Preparation of E. coli competent cells

In order to investigate the CM11 peptide influences on the ability of *E. coli* to uptake foreign DNA, two methods were used to prepare the competent cells. In the first method, *E. coli* was refreshed for 14 hrs in presence of 0.5, 1, 2, 3 and 6 μg/mL of CM11 peptide. Then five separate tubes containing 5 mL Luria-Bertani (LB) broth medium (Merck) supplemented with 0.5, 1, 2, 3 and 6 μg/mL CM11 peptide were inoculated by refreshed *E. coli* and incubated at 37 °C and 150 rpm to an optical density of 0.5 at 600 nm. Then cells were harvested at 3000 g for 5 min. Cell pellets obtained from media containing different concentrations of peptide in separate tubes, were suspended with 4 mL of cold 50 mM $CaCl_2$ along with equivalent peptide concentrations and were kept in ice for 1 hr. Afterwards, tubes were centrifuged at 3000g for 5 min and obtained pellets containing competent cells. Cells resuspended in 500 μL cold 50 mM $CaCl_2$ with equivalent peptide concentrations.

In the second method, *E. coli* was refreshed without peptide. Then 5 mL Luria-Bertani broth medium was inoculated by refreshed *E. coli* and incubated at 37 °C and 150 rpm to an optical density of 0.5 at 600 nm. Then cells were centrifuged at 3000 g for 5 min and pellets were suspended with 4 m of cold 50 mM $CaCl_2$ and kept in ice for 1 hr. The cell suspension was centrifuged at 3000 g for 5 min and obtained pellet was resuspended in 500 μL cold 50 mM $CaCl_2$. Afterwards, Cells were equally divided into five separate tubes containing of 0.5, 1, 2, 3 and 6 μg/mL of CM11 peptide as describe above.

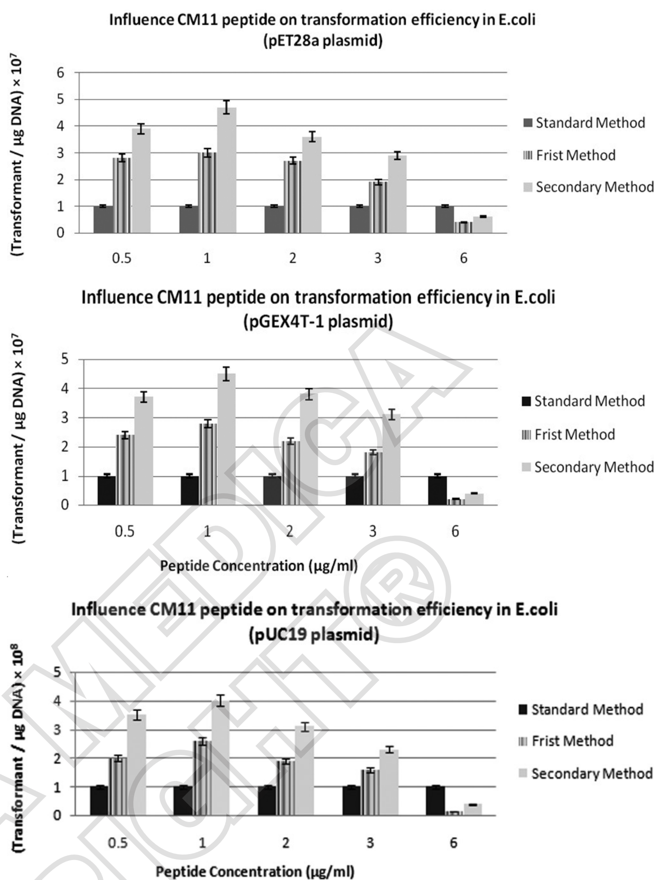

Figure 2.—Plasmid transformation efficiency in *Escherichia coli* (as a Gram-negative bacteria) using CM11 peptide. *E. coli* transformed with three plasmids pET28a(+) (2.A), pGEX4T-1 (2.B), and pUC19 (2.C) at different peptide concentration by three methods. The X-axis represents the different peptide concentration, and the Y-axis represents the transformation efficiency. Tests were performed 3 times, and the means and standard deviations are indicates a statistically significant difference transformation rates.

Preparation of B. subtilis competent cells

In order to investigate the CM11 peptide influences on the ability of *B. subtilis* to uptake foreign DNA, two methods were used to prepare the competent cells. In the first method, the strain was refreshed on Luria-Bertani agar medium. Afterwards, five sterile vial containing 1 mL spizizen broth medium supplemented with 0.5, 1, 2, 3 and 6 μg/mL of CM11 peptide were inoculated by one colony form of overnight agar culture and incubated at 35 °C and 150 rpm to an optical density of 0.5 at 600 nm.[20]

In the second method, preparation of bacteria was performed without peptide in spizizen medium

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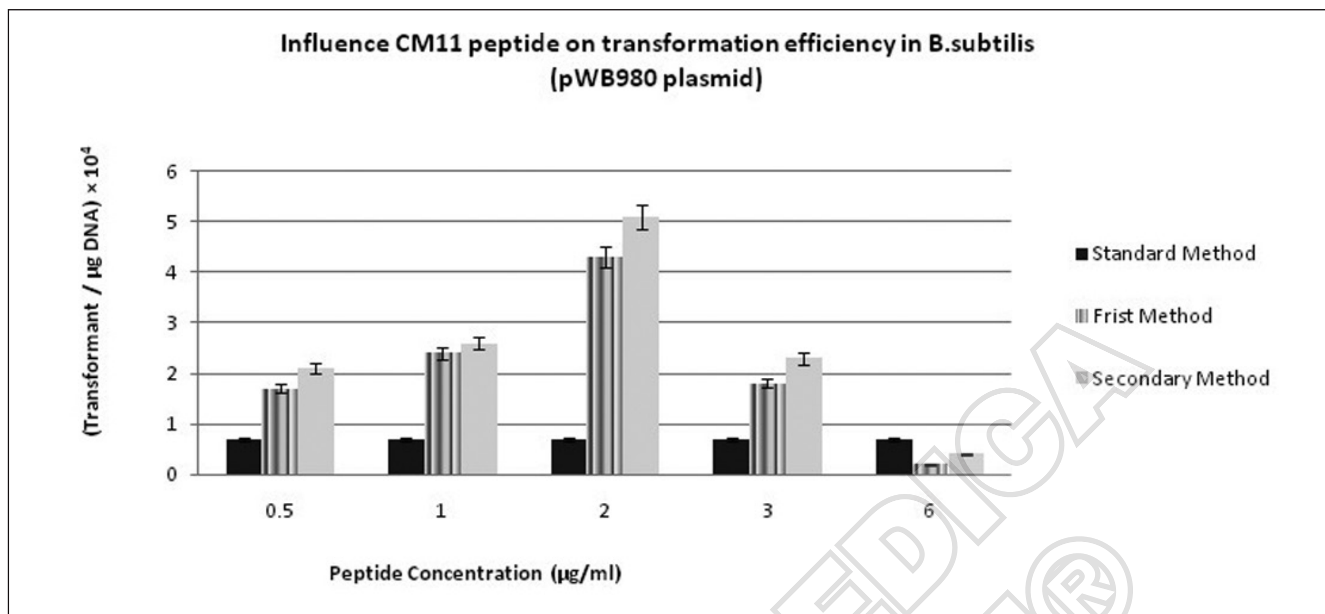

Figure 3.—Plasmid transformation efficiency in *Bacillus subtilis* (as a gramGram-positive bacteria) WB600 using CM11 peptide. *B.subtilis* WB600 transformed with pWB980 plasmid at different peptide concentration by three methods. The X-axis represents the different peptide concentration, and the Y-axis represents the transformation efficiency. Tests were performed 3 times, and the means and standard deviations are indicates a statistically significant difference transformation rates.

and peptide was added along with plasmid during transformation step.

E. coli BL21 transformation

Separately and under sterile conditions, 100 µL of *E. coli* BL21 (DE3) pLysS competent cells that prepared by each method, were transferred into ten vials with 1.5 mL of volume (5 vials for first and 5 vials for second method). Then 1 µL of 100 ng/µL pET-28a(+) plasmid, 2 µL of 50 ng/µL pGEX4T-1 plasmid and 1 µL of 100 ng/µL pUC19 plasmid were separately added into ten individual vials. Subsequentthe common method was performed to transform Gram-negative bacteria.[21] After transformation, in order to detect the amount of pET-28a(+), pGEX4T-1 and pUC19 plasmids received by bacteria, 1 µL of harvested cells were cultured on LB agar containing 80 µg/mL kanamycin (for pET-28a(+)) and 100 µg/mL ampicillin (for pGEX4T-1 and pUC19 plasmids).

B. subtilis WB600 transformation

To transform *B. subtilis* WB600 by first method, 10 µL of 100 ng/µL pWB980 plasmid was added to each five vials of 1 mL spizizen medium containing competent bacterial cells and different amounts of peptide. Then vials were incubated at 35°C for 14 hrs. In the second method, which transformation was simultaneously performed with peptide treatment, five different vials of 1 mL spizizen medium containing competent bacteria were diluted with different concentration of peptide (0.5, 1, 2, 3 and 6 µg/mL) and 10 µL of 100 ng/µL pWB980 plasmid, then vials incubated at 35 °C for 14 hrs. Afterwards, the pellets were obtained from centrifugation at 3000g for 5 min which were resuspended in 20 µL fresh medium and cultured on LB agar with 30 µg/mL kanamycin. It is necessary to prepare control for both bacterial samples in order to comparison between treated and untreated. In control sample all steps of competent cell processing and transformation were performed in the absence of peptide. The experiments were performed three times to check there peat ability.

Screening of bacteria transformed with plasmid

Bacterial transformation confirmed by growing *E. coli* and *B. subtilis* bacteria on LB agar plates con-

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TABLE I.—*Plan for transformation of plasmids and calculation of its efficiency.*

	Efficiency calculator				*E. coli plasmids*			*B. subtillis plasmid*
					pET28a(+)	pGEX4T-1	pUC19	pWB980
1	Concentration (μg/μL) of the DNA used to transform				1	0.5	1	1
2	Volume (μL) of DNA added to the transformation mix				1	2	1	1
3	Total volume (μL) of competent cell+ DNA+ recovery medium (transformation mix)				100+1+1000	100+2+1000	100+1+1000	100+1+1000
4	Volume (μL) actually plated from the transformation mix (for a single plate)				10	10	10	all
5	Mean of colonies or transformants from the plate above of 3 times	First method	Peptide concentration	0.5 μg/mL	235	198	171	16
				1 μg/mL	254	229	216	21
				2 μg/mL	220	180	161	38
				3 μg/mL	155	147	135	16
				6 μg/mL	32	16	12	2
		Second method	Peptide concentration	0.5 μg/mL	322	304	287	20
				1 μg/mL	384	360	332	23
				2 μg/mL	294	281	256	46
				3 μg/mL	237	244	185	21
				6 μg/mL	49	36	33	3
		Standard		No- peptide	79	88	95	8

tains ampicillin and kanamycine. Also plasmid extraction was used in order to verification of transformation.

Bacteria transformation efficiency

In order to measure the effectiveness of the peptide, the experiments were performed acording to variable parameters listed in Table I, then quality of transformation efficiency were evaluated by using results and below formula. To test the transformation efficiency of competent cells from each method, each experiment was repeated three times to calculate the average value as the transformation efficiency.

$$\text{Transformation efficiency} = \frac{\textit{Total of clony (E.coli or B.subtillis) on LB agar}}{\textit{Amount of DNA concentration } (\frac{\mu g}{ml})}$$

Statistical analysis

Descriptive statistics are reported as means and standard deviations (mean ±SD). An independent *t*-test was considered to compare the scores of each of the measures and some of the parameters data between the two groups. The ANOVA model was utilized for statistical analysis of parameters between all groups. A probability of less than 0.05 was considered statistically significant. Data were analyzed using SPSS, version 16 (FAQs). The data in each figure was a representative of tree independent

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experiments expressed as the mean ± standard deviation (SD).

Results

In order to evaluate the effectiveness of various concentrations of peptide on plasmid uptake by *E. coli*, results were investigated after 14 hrs culturing of transformed *E. coli* on the selective LB agar using colony counts and after calculating the transformation efficiency of plasmid, results were compared to control samples (CaCl2 standard method). Studies have shown that the transformation efficiency of transformed *E. coli* by pET28a(+) was increased in presence of 1 µg/mL peptide using both different methods, which were ~3-fold and ~5-fold grater than control sample, respectively (Figure 2.A). Statistical analysis of results for both methods showed that the observed difference is too large (P<0.001). By studying more cases, results showed that increasing concentration of CM11 peptide above 1 µg/mL causes the decrease in the number of transformed bacteria. So that at the presence of 6 µg/mL peptide, the number of transformed bacteria was significantly lower than control (P=0.014 and P=0.04 orderly for first and second method). The same results were obtained using pGEX4T-1 plasmid; so that the competent cells prepared with 1 µg/mL peptide, showed respectively ~3 fold and ~5 fold greater amounts of transformtion than ontrol using first and second methods, (Figure 2.B). Confirmation of statistical analysis showed the significance of the results (P<0.001). The inverse relationship between rate of transformation and peptide concentration was also repeated at the presence of 6 µg/mL of peptide (P<0.001 and P=0.016 for first and second methods).

For pUC19 plasmid; the best result obtained with using competent cells that prepared with 1 µg/mL peptide. In this concentration the rate of transformation were respectively ~3 fold and 4 fold for first ad second methods compared with control (Figure 2.C). Also statistical analysis proved the different significant in results (P<0.001). Also for all plasmids, results showed that the transformation rate at the presence of 0.5 and 2 µg/mL peptide concentrations was the same.

In general, *B. subtilis* transformation is inefficient and occurs at a low rate but using peptide can improve the yield of pWB980 plasmid transformation. As shown in Figure 3, transformation efficiency of *B. subtilis* by first method and using 2 µg/mL peptide concentration was 4.2 × 104 cfu/µg equal to ~5-fold of control. Also the results indicated that preparing *B. subtilis* competent cells using the second method with similar peptide concentration has the maximum efficiency (5.1×10^4 cfu/µg, about 7-fold of control). Results showed that the transformation rate at the presence of 0.5 and 3 µg/mL peptide concentrations was the same. Statistical analysis of data from both methods showed a significant difference between test and control sample (P<0.001). Like *E. coli* test, a meaningful decrease in rate of transformation with increase of peptide concentration was observed (P<0.001).

Discussion

In biological research laboratories the most widely used methods for DNA transformation of bacteria are chemical ($CaCl_2$) and electroporation transformation, however, both methods require preparation of competent cells and recovery. On the other hand, electroporation technology is limited in disadvantage of preparation of large quantities of cells and DNA, also special equipments is required that many laboratories cannot provide them. But, the chemical methods have attained much attention in most of the laboratories, due to their accessibility and cost effectiveness.[22]

In recent years many studies have been done to improve the conventional methods of DNA transfer or provide new methods. In this regard, Yoshida *et al.* (2001) reported a novel transformation method based on mineral nanofibers that improved by Wilharm *et al.* (2010).[23, 24]

However, Tan *et al.* (2010) confronted many problems trying this newest method.[25] According to their investigation, only a few transformants could be obtained even by using 100 ng of plasmid and a successful result seemed difficult to repeat. They changed the operating method and thereby enhanced the transformation efficiency greatly trying plasmid transformation based on carbon nanotubes (CNTs) and got more than 15,000 transformants per 100 ng plasmd compared with calcium chloride transformation (8,000 transformants per 100 ng of plasmid). Although the results of this method is considerable but now the widespread use of nanotubes as a new method is associated with restrictions that seems not to be appropriate for the future.

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Zhiming Tu *et al.* (2005) described the use of different *E. coli* strains for optimization of plasmid transformation using $CaCl_2$ chemical method. Based on this method, they have established an efficient system using *E. coli* competent cells for transformation of plasmids.[26] They checked some factors affected the yield of plasmid transformation such as OD_{600} of culture medium, concentration of $CaCl_2$ and composition of bacterial culture medium for three *E.coli* strains (DH5α, TG, XL1 blue). The results showed that their improved bacterial transformation system can raise the transformation efficiency about 10^3 times. In their study the best results were obtained for XL1 blue bacteria strain. In this study a point is noteworthy: working with DH5α is cheaper and comprehensive in research laboratories but, on average, the highest rate for the transfer plasmid (for five plasmids) to the bacteria strains obtained for XL1 blue strain (~8×10^8) while the lowest transmission is for DH5α bacteria strain (~4×10^8).

In another study, Singh *et al.* (2010) analyzed various parameters of standard $CaCl_2$/heat shock method for transformation of *E. coli* strain DH5α -T1R with plasmid pUC19 to optimization of this method. Based on the results, they suggested that a heat shock pulse of 30 sec at 42°C followed by 10 minutes ice incubation step are ideal parameters to obtain maximum transformation efficiency in DH5α -T1R strain. Also they suggested that post heat shock cold incubation step is also an important factor and enhances transformation of *E. coli* significantly.[27]

In the present study, as working with DH5α and $CaCl_2$ chemical method are usual in many research laboratories, we investigated a manner to improve this method by cell permeable peptides.

For this purpose we used the CM11 cationic peptide as a cell permeable peptide. CM11 like other cationic and amphipathic peptides has the ability of pore formation in bacterial membranes and permeabilization of cytoplasmic membrane. According to studies by Yechiel Shai, since the 11-residue hybrid peptide CM11 is not long enough to span the entire width of the membrane for pore formation, a "carpet-like" mechanism seems to be more plausible.[28, 29] In this model, interactions between negatively charged phospholipid and cationic CPPs result in a carpeting and thinning of the membrane, respectively. According to this mechanism, at a critical threshold concentration the peptides form toroidal transient holes in the membrane and above this concentration; the membrane disintegrates and forms micelles after disruption of the bilayer curvature.[29, 30]

X-ray analysis has shown that peptide binding caused membrane thinning but pores appeared only when the thinning reached a critical fraction of the membrane thickness and it is noticeable that binding and pore formation potencies of these peptides are separate events. Also, antimicrobial activities of these peptides typically exhibit sigmoidal concentration dependence so below the threshold concentration, there is little activity despite binding but as the concentration exceeds the threshold, the activity rapidly reaches its maximum level which leads to bacterial death.[29] It seems that in low concentration, peptide has potent outer membrane-disorganizing but a rather weak damaging action on cytoplasmic membrane. In higher concentration, peptide will allow access to the cytoplasmic membrane, where peptide pore formation has been proposed to occur which results in increasing of permeability for some external and internal compounds, which can ultimately lead to death (upper MIC).[30] Previous studies showed MIC of peptide for *E.coli* and *B.subtilis* in most instances is 8 µg/mL, this peptide concentration may actually be the threshold concentration for the peptide.[31]

Based on this findings and MIC of CM11 peptide for *E.coli* and *B.sbtillis,* we investigated the effect of CM11 peptide on plasmid transformation efficiency in these bacteria. Findings by Singh *et al.* were used as fundamental condition in this study and all results were comprised with this control model.[27]

The results showed that the highest amount of transformed *E.coli* is occurred in 1 µg/mL as a moderate peptide concentration and with increasing peptide concentration the number of transformed bacteria was declined. This is a little difference in Gram-positive bacteria such as *B. subtillis*, because the highest amount of transformation is performed at 2 µg/mL concentration. It seems that this variation is to differentiation between the cell membrane of Gram-positive and Gram-nege bacteria. The thick layer of peptidoglycan in Gram-positive bacteria acts as a barrier for peptide to access the double phospholipids membrane and with increasing the concentration of peptide, the access coefficient to the membrane rises and consequently increases the efficiency of DNA transformation. Also, since this peptide has antimicrobial activity, increasing the peptide concentration leads to an increase of its lethal effects on both types of bacteria. Of course

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the lethal rate of peptide depends on the amount of MIC, so that the closer concentration of peptide to MIC increases the amount of lethal effect. Based on this theory and obtained results, it can be justified that in bacteria which were exposed to the peptide longer in the first method, the transformation rate was reduced equivalently due to the increase of lethal rate. Accordingly, the results showed that bacteria exposed to concentrations of 3 and 6 µg/mL have a lower transformation efficiency compared to the concentrations of 1 and 2 µg/mL for *E. coli* and *B. subtillis*, respectively. Above these optimum concentrations there is an inverse relationship between peptide concentration and transformation rate because higher concentrations are close to MIC. In 6 µg/mL concentration which is closest to the MIC, transformation rate is even lower than the standard sample that might be as the result of reaching of peptide concentration to the threshold point, which leads to the complete disintegration of membrane structure; therefore optimal peptide concentration is considered very important. Also results showed that there is not any differentiation between 0.5 and 2 µg/mL peptide concentrations in *E.coli* plasmid transformation rate. About *B. subtillis* this undifferentiating result was done between 0.5 and 3 µg/mL of peptide concentrations. It seems that undifferentiating in transformation rate is due to a balance between DNA uptake severity and bacteria killing by peptide i.e. although in 0.5 µg/mL concentration, DNA uptake is lower than uptake in 2 and 3 µg/mL concentrations (For *E. coli* and *B. subtillis*, respectively) but a bacterium killing occur in a lower rate by peptide.

Our studies have shown that the peptide alone will not be able to facilitate the transfer of DNA within the bacterial cell. Therefore, the experiments were designed during this study to evaluate the ability of the peptide in facilitating the DNA transformation without $CaCl_2$ and heat shock, which didn't have desirable results (results not shown). It seems that the presence of peptide leads to an increasing transformation efficiency of bacteria just by changes in the arrangement of membrane structure and so increase of its permeability to DNA, without considerable interaction with DNA.

Finally, in this study results have shown that the pUC19 plasmid transformation rate (4.1×10^8 cfu/µg DNA) is 4 fold higher than its control (0.9×10^8) and on the other hand the pET and pGEX plasmid transformation rates (4.7×10^7 and 4.4×10^7 cfu/µg DNA) are 4.7 and 4.4 fold higher than controls (1.1×10^7). For pUC19 plasmid constant coefficient of transformation rate in comparison with control is 10^8 whereas for pET28 and pGEX4T-1 plasmids this coefficient is 10^7, so these results show an inverse relationship between plasmid size and transformation rate.

Conclusions

These studies showed that CM11 cationic peptide as a cell permeable peptide can increase the plasmid transformation efficiency in bacterial cells. So these results can be used as an initial and basic data for some more studies on these types of peptides in order to further increase transformation efficiency in bacteria.

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Acknowledgements.—The authors wish to thank all the colleagues in the Applied Biotechnology Research Center for their kind help during the research.

Conflicts of interest.—The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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